Novel antibacterial calcium phosphate nanocomposite with long-term ion recharge and re-release to inhibit caries

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Short-term studies on calcium-phosphate (CaP) ion-rechargeable composites were reported. The long-term rechargeability is important but unknown. The objectives of this study were to investigate nanocomposite with strong antibacterial and ion-recharge capabilities containing dimethylaminododecyl methacrylate (DMAHDM) and nanoparticles of amorphous calcium phosphate (NACP), and evaluate long-term ion-recharge by testing for 12 cycles (taking 6 months to complete) for the first time. Three groups were tested: (1) Heliomolar control; (2) Resin+20%NACP+50%glass; (3) Resin+3%DMAHDM+20%NACP+50%glass. Biofilm acid and colony-forming units (CFU) were measured. Ion-recharge was tested for 12 cycles. NACP-DMAHDM composite reduced biofilm acid, and reduced CFU by 4 logs. High levels of ion releases were maintained throughout 12 cycles of recharge, maintaining steady-state releases without reduction in 6 months (p>0.1), representing long-term remineralization potential. Bioactive nanocomposite demonstrated long-term ion-rechargeability for the first time, showed remineralization and potent anti-biofilm functions, with promise for tooth restorations to combat caries.

Keywords: Dental composite, Calcium phosphate nanoparticles, Ion recharge and re-release, Dimethylaminododecyl methacrylate, Dental caries

INTRODUCTION

Resin composites are the most frequently used direct restorative material due to their conservative removal of tooth structure, improved esthetics, and directfilling capabilities^{1,2)}. However, composites still present some serious drawbacks, including recurrent caries, restoration fracture, and marginal leakage³⁾. Marginal leakage allows for bacterial entry, proliferation, and production of acids, subsequently resulting in recurrent caries. Moreover, composites favor the accumulation of biofilms/plaque^{4,5)} when compared to other restorative materials⁴). Previous studies have shown that plaque associated with composite restorations tends to favor the adherence of the more cariogenic bacteria, including mutans streptococci and lactobacilli6). Composites can accumulate more biofilm leading to tooth structure demineralization and recurrent caries at the toothrestoration interface, which is 3.5 times more common with composites than with amalgam⁷). Recurrent caries is considered one of the two main reasons to replace an existing restoration^{3,8)}, followed by restoration

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fracture^{9,10)}. The replacement of failed dental restorations constitutes approximately 50 to 70% of performed operative dental procedures^{9,11)}, which involves removal of additional tooth structure, resulting in a weaker tooth that is more susceptible to fracture¹²⁾.

Therefore, there is a strong need to develop a new generation of bioactive dental composites with antibacterial and remineralization abilities^{8,13)}. Quaternary ammonium methacrylates (QAMs) were synthesized and incorporated into resins to produce antibacterial effects¹⁴⁻¹⁶⁾. QAMs are a class of cationic compounds with a broad-spectrum antimicrobial effect^{17,18}). The alkyl chain length (CL) of quaternary ammonium compounds has been correlated with effectiveness¹⁹⁻²²⁾. their antibacterial Recently, dimethylaminohexadecyl methacrylate (DMAHDM) with a CL of 16 was synthesized and shown to have stronger antibacterial properties than QAMs with shorter CLs²⁰. DMAHDM has been incorporated into composites and adhesives that showed potent antibacterial properties against a wide range of oral pathogens^{20,21,23-26}).

Another way to combat dental caries is through the application of remineralizing materials that can release calcium (Ca) and phosphate (P) ions, which constitute

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the major mineral component of the tooth structure. Nanoparticles of amorphous calcium phosphate (NACP) were synthesized and previously incorporated into composites that promoted tooth remineralization through the release of high levels of Ca and P ions²⁷⁾ especially in challenging acidic environments where they are mostneeded²⁸). NACP composite had the capability of being repeatedly recharged to provide ion re-release, thereby demonstrating a long-term remineralization potential^{29,30}. A previous study showed the rechargeability of Ca and P ions for a NACP containing composite through six cycles of recharge and re-release taking 48 days to complete²⁹⁾. However, that rechargeable NACP composite had no antibacterial properties³⁰⁾. The rechargeability of proteinrepellant adhesive³¹⁾, protein-repellant nanocomposite³²⁾ and antibacterial EBPM composite²⁹⁾ were investigated through only three cycles of recharge and re-release cycles taking 45 days to complete. To date, there has been no report on the long-term Ca and P ion recharge and re-release capabilities that extend beyond 6 cycles of recharge and re-release with time periods longer than 48 days.

The objective of this study was to investigate the longterm Ca and P ion release and remineralization potential of a bioactive NACP-DMAHDM composite by evaluating 12 cycles of recharge and re-release which took 6 months to complete, for the first time. It was hypothesized that: (1) The long-term Ca and P ion recharge and re-release would not be compromised by the addition of DMAHDM; (2) The rechargeable NACP-DMAHDM composite would have much less biofilm growth and lactic acid production, compared to groups without DMAHDM; (3) The new bioactive composite would provide long-term remineralization with maintained Ca and P ion release throughout the 12 cycles of recharge and re-release.

MATERIALS AND METHODS

Fabrication of composite containing DMAHDM

The resin matrix consisted of 49.5% ethoxylated bisphenol A dimethacrylate (EBPADMA; Esstech, Essington, PA, USA) and 49.5% pyromellitic glycerol dimethacrylate (PMGDM; Esstech) (all mass % unless specified otherwise). In addition, 0.2% camphorquinone and 0.8% ethyl 4-N,N-diethylaminobenzoate were added for light-cure activation. This resin was referred to as EBPM³³.

DMAHDM was synthesized using a modified Menschutkin reaction, in which a tertiary amine group was reacted with an organ-halide³⁴⁾. Briefly, 10 mmol of 2-(dimethylamino) ethyl methacrylate (DMAEMA; Sigma-Aldrich, St. Louis, MO, USA), 10 mmol of 1-bromododecane (BDD, TCI America, Portland, OR, USA), and 3 g of ethanol were combined in a vial, which was stirred at 70°C to react for 24 h. When the reaction was completed, the ethanol solvent was eliminated by evaporation to yield a colorless, clear and viscous DMAHDM liquid²²⁾. DMAHDM was incorporated into EBPM at a mass fraction of DMAHDM/ (EBPM+DMAHDM)=10%. A total filler mass fraction of 70% was incorporated into the resin matrix as described below, thus resulting in a 3% DMAHDM mass fraction in the final composite, following a previous study²².

A spray-drying technique was used to synthesize NACP by dissolving 0.8 g of calcium carbonate and 5.094 g of di-calcium phosphate anhydrous in 1.5125 g of acetic acid. The Ca and P ionic concentrations were 8 and 5.333 mmol/L, respectively. This solution was sprayed into a heated chamber of the spray-drying machine, and then an electrostatic precipitator was used to collect the dried NACP particles. The NACP had a Ca/P molar ratio of 1.5, similar to that of ACP [Ca₃(PO₄)₂]²⁸. The mean particle size was 116 nm²⁸.

Silanized barium boroaluminosilicate glass particles with a median size of 1.4 μ m (Caulk/Dentsply, Milford, DE, USA) were used for mechanical reinforcement of the composite. The total filler mass fraction in the resin was 70%, including 20% NACP and 50% glass, which were readily mixed into the resin mixture using a Speed-Mixer (DAC 150.1, FlackTek, Landrum, SC, USA) to produce a cohesive composite paste.

A commercial composite (Heliomolar, Ivoclar, Ontario, Canada) was used as a comparative control. It contained 66.7% nano-fillers of 40 to 200 nm of silica and ytterbium-trifluoride with fluoride-releasing capabilities.

Three groups of composites were included in the subsequent experiments:

- Heliomolar (referred to as commercial control composite);
- (2) Rechargeable NACP composite: 30% EBPM+20% NACP+50% glass (referred to as Rechargeable NACP);
- (3) Rechargeable antibacterial NACP-DMAHDM composite: 27% EBPM+3% DMAHDM+20% NACP+50% glass (referred to as Rechargeable NACP-DMAHDM).

Flexural strength and modulus of elasticity

Stainless steel molds of $2 \times 2 \times 25$ mm were used to fabricate samples for measurements of flexural strength and modulus of elasticity. Each composite paste was placed into a mold, covered with Mylar strips and light-cured (Triad 2000, Dentsply, York, PA, USA) for 1 min on each side. The irradiance of the Triad 2000 curing unit was previously calculated to be 65 mW/cm^{2 35)}. Specimens were incubated in an oven at 37°C for 24 h. Flexural strength and elastic modulus were measured using a three-point flexural test with a 10 mm span at a crosshead-speed of 1 mm/min on a computer-controlled Universal Testing Machine (5500R, MTS, Cary, NC, USA)^{28,36}. Flexural strength S was calculated by: $S=3P_{max}L/(2bh^2)$, where P_{max} is fracture load, L is span, b is specimen width and h is specimen thickness. Elastic modulus E was calculated by: E=(P/d) ($L^{3}/[4bh^{3}]$), where load P divided by displacement *d* is the slope in the linear elastic region of the load-displacement curve^{28,36)}.

Composite surface roughness

Composite bars with approximately 2×2×12 mm

dimensions were used for surface roughness assessments before starting the ion release and recharge. The average surface roughness (R_a , μ m) was measured with a Mitutoyo (SJ-310 SURFTEST, Kanagawa, Japan) surface roughness tester following a previous study (n=6)³⁷. In a previous study, composite surfaces produced using Mylar strips showed surface roughness values that were not significantly different from the Enhance/BisCover finishing protocol³⁸). Both the Enhance/BisCover and the Mylar strip-formed surfaces were much smoother than other surfaces produced by various finishing and polishing procedures³⁸). Therefore, in the present study, the surface roughness measurement was done on composite surfaces produced using Mylar strips. The surface roughness device had a 5 μ m stylus tip radius.

Samples for biofilm testing

Sample disks were fabricated using molds with a diameter of 9 mm and thickness of 2 mm. Disks were light-cured (Triad 2000, Dentsply) for 1 min on each side²¹). The cured disks were immersed in distilled water and magnetically stirred at 100 rpm for 1 h to remove the initial burst of uncured monomers, following previous studies^{24,39}. The disks were then sterilized with ethylene oxide (AnproleneAN 74i, Andersen, Haw River, NC, USA) and degassed for 7 days following the manufacturer's instructions.

Bacteria inoculation and biofilm formation

The bacterial experiments were approved by the University of Maryland, Baltimore Institutional Review Board. S. mutans biofilm was selected in the current study because S. mutans is one of the main pathogenic bacteria highly associated with secondary caries formation around composite restorations⁴⁰. S. mutans showed its ability to create an aggressive plaque biofilm and acidify the local plaque environment, all of which contributed to caries formation^{41,42)}. S. mutans was obtained from the American Type Culture Collection (ATCC 700610, UA159, Manassas, VA, USA). S. mutans was inoculated in brain heart infusion broth (BHI, Difco, Sparks, MD, USA) at 37°C under aerobic condition (5% CO₂) for 16 h and then adjusted to 10^7 colony-forming units (CFU)/ mL using a spectrophotometer (Genesys 10S, Thermo Scientific, Waltham, MA, USA) based on the OD 600 nm versus CFU/mL. This adjusted suspension was used as the inoculum.

Composite disks were placed in a 24-well plate filled with 1.5 mL of BHI supplemented with 1% sucrose and the adjusted *S. mutans* inoculum. After 24 h, the disks with adherent biofilm were transferred to a new 24-well plate filled with fresh medium, according to a previous study⁴³. Biofilms on disks were grown for 48 h to form relatively mature biofilms.

Live/dead staining assay

Composite disks with 48 h biofilm were gently washed three times with phosphate buffered saline (PBS), then stained with Live/Dead Baclight bacterial viability kits following the manufacturer's instructions (Molecular Probes, Eugene, OR, USA)^{20,44)}. SYTO 9 and propidium iodide were mixed at 1:1 ratio and used to stain the disks for 15 min. Live bacteria were stained with SYTO 9 to radiate a green fluorescence. Dead bacteria with compromised cell membrane were stained with propidium iodide to radiate a red fluorescence.

Images of stained disks were captured with an inverted epifluorescence microscope (TE2000-S, Nikon, Melville, NY, USA). The images were captured with the red and green fluorescence filters separately and then overlaid to become a single image. The time and location of the captured images (green and red fluorescence) were kept constant. Three composite disks before ion release and recharge were tested for each group.

In addition, to assess the antibacterial activity after the 12 cycles of recharge and re-release, the composite bars from the recharge and re-release tests were used for bacterial viability via the Live/dead staining assay (n=4) for the rechargeable NACP composite and the rechargeable NACP-DMAHDM composite. After the 12 cycles, the composite bars were sterilized with ethylene oxide (AnproleneAN 74i) and degassed for 7 days following the manufacturer's instructions. S. mutans inoculum in brain heart infusion broth was adjusted on OD 600 to 107 colony-forming units (CFU)/mL using a spectrophotometer (Genesys 10S). The composite bars were placed in a 24-well plate filled with 1.5 mL of inoculum. After 24 h, the bars with adherent biofilm were transferred to a new 24-well plate filled with fresh medium and cultured for another 24 h, totaling 2 days of culture. The 2-day biofilms on composites were used for live/dead staining.

Biofilm CFU counts

Disks with 2-day biofilms were transferred into a new 24-well plate filled with 1 mL PBS. The biofilm was harvested by scraping and sonication/vortexing (Fisher, Pittsburg, PA, USA), according to previous studies^{20,21)}. The suspensions were serially diluted and spread on BHI agar plates to evaluate the number of *S. mutans* colonies. After 48 h incubation at 37°C in 5% CO₂, the number of colonies was counted and used with the dilution factor to determine the CFU counts.

Lactic acid production by biofilms

Disks with 2-day biofilms were washed three times with PBS, and then immersed in a 24-well plate filled with 1.5 mL of buffered peptone water (BPW; Sigma-Aldrich) supplemented with 0.2% sucrose. The 24-well plate was incubated at 37°C in 5% CO₂ for 3 h^{21,22)}. The lactate concentrations produced by biofilms were determined using an enzymatic method *via* the lactate dehydrogenase approach. This was accomplished by measuring the absorbance at an OD 340 nm using a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA) with known lactic acid standards (Supelco Analytical, Bellefonte, PA, USA) and calibration curves, according to previous studies^{21,22)} (*n*=5). For ion release measurements, only group 2 and 3 were tested for Ca and P ion release, the commercial control composite was not included as it is not expected to release Ca and P ions. To simulate a cariogenic condition in the oral cavity, a sodium chloride (NaCl) solution (133 mmol/L) buffered to pH 4 using 50 mmol/L lactic acid was used to measure the ion $release^{28,45}$. Three specimens of approximately 2×2×12 mm were immersed in 50 mL of NaCl solution. This had a specimen volume/ solution of 2.9 mm³/mL, following previous studies^{29,46}. Ca and P ion concentrations released from the specimens were measured at 1, 3, 5, 7, 14, 21, 28, 35, 42, 49, 56, 63 and $70~{\rm days^{29,46)}}.$ At each time point, aliquots of 0.5mL were removed and replaced by fresh solution³⁰. The aliquots were analyzed for Ca and P ion concentrations using a spectrophotometric method (DMS-80 UV-vis, Varian, Palo Alto, CA, USA) via known standards and calibration curves $(n=8)^{30,46}$. The measured ion releases are stated as the "initial release", to distinguish it from the recharge and re-release in the section below.

Ca and P ion recharge and re-release

After the completion of the initial ion release for 70 days, the same specimens were used for the recharge and rerelease test. The Ca ion recharge solution contained 20 mmol/L CaCl₂ and 50 mmol/L of 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) buffer, while the P ion recharge solution contained 12 mmol/L KHPO₄ and 50 mmol/L HEPES buffer. Both solutions were adjusted to pH 7 using 1 mol/L KOH⁴⁷). Three specimens of approximately $2\times2\times12$ mm were immersed in 5 mL of the Ca or P solution and gently agitated for 1 min on a vortex machine (Analog Vortex Mixer, Fisher Scientific, Waltham, MA, USA) at a power level of 3 in order to simulate the mouthwash usage action. Then the specimens were rinsed with distilled water for 1 min to remove any loosely attached ion deposits on specimen surfaces. Following previous studies, the recharge was repeated three times at 9:00 am, 1:00 pm and 5:00 pm for one day, for a total of 3 min of recharge^{30,48)}. Then these recharged specimens were immersed into a 50 mL NaCl (133 mmol/L) solution at pH 4 to measure the ion rereleases at 1, 3, 5, 7, 9 and 14 days^{29,30)}. This constituted one cycle of recharge and re-release. The second cycle was then started by recharging the samples for another 3 min, and the ion re-releases were measured for another 14 days. Each cycle took 15 days, with the test of 12 cycles taking nearly half a year to complete.

Statistical analysis

Statistical analyses were performed with SPSS, version 25.0 (SPSS, Chicago, IL, USA) at an alpha of 0.05. Oneway analysis of variance (ANOVA) and Tukey's multiple comparison tests were used to detect the significant effects of the variables. *T*-test was used for the analysis of Ca and P initial ion release and recharge and re-release. Each error bar represents a standard deviation (SD) and serves as the estimate for measurement uncertainty.

RESULTS

Figures 1A and B plots the flexural strength and modulus of elasticity of the three groups (mean±SD; n=6). The three composites had equivalent mechanical properties (p>0.05). The rechargeable NACP-DMAHDM composite showed no significant difference in flexural strength and modulus of elasticity when compared to those without DMAHDM (p>0.05). This indicates that adding DMAHDM and NACP did not compromise the mechanical properties.

Figure 1C plots the composite surface roughness (mean±SD; n=6). Adding 3% DMAHDM did not significantly affect the roughness of the composite, compared to that without DMAHDM (p>0.05). Both





(A) Flexural strength, (B) elastic modulus, and (C) Surface roughness of composites (mean \pm SD; n=6). Adding 3% DMAHDM did not significantly affect the flexural strength, modulus of elasticity and the surface roughness of the composite, compared to that without DMAHDM (p>0.1). Both the rechargeable composites matched the properties of commercial control (p>0.1).

rechargeable composites had a surface roughness matching that of the commercial control composite (p>0.05).

Representative live/dead images of 2-day biofilms on the composites are presented in Fig. 2. Both the commercial control and the rechargeable-NACP composite with 0% DMAHDM were mostly covered by live bacteria as represented by the green color. On the other hand, the rechargeable NACP-DMAHDM composite showed less live bacteria, and was mostly covered by dead bacteria, represented by the red color.

The CFU counts are plotted in Fig. 3A for 2-day biofilms on composites (mean \pm SD; *n*=5). The CFU counts on disks containing DMAHDM were about 3 to 4 orders of magnitude less than those without DMAHDM.

The rechargeable NACP-DMAHDM composites were the most effective in reducing biofilms when compared to the commercial control and the rechargeable NACP composite without DMAHDM (p<0.05).

The level of bacterial lactic acid production is plotted in Fig. 3B (mean±SD; n=5). Both the commercial control and the rechargeable-NACP composite with 0% DMAHDM had a similar metabolic activity and high lactic acid production (p>0.1). In comparison, the rechargeable NACP-DMAHDM composite showed significant reduction in lactic acid production of biofilms (p<0.05).

Figure 4 presented the initial ion release of Ca and P ions (mean±SD; *n*=8): (A) Ca ion release, and (B) P ion release. There was no significant difference in Ca and P



Fig. 2 Representative live/dead staining images of 48 h biofilms on composites. (A and B) Composite specimens without DMAHDM were fully covered by primarily live bacteria. (C) In comparison, NACP-DMAHDM composite had markedly less live biofilms, and the biofilms presents mainly compromised bacteria.



Fig. 3 Biofilm growth on composites.

(A) Colony-forming unit (CFU) counts, and (B) Lactic acid production of 48 h biofilms on composites (mean±SD; n=5). Biofilm CFU counts of 3% DMAHDM group were approximately 3 to 4 orders of magnitude lower than those on the other two composites. Values with dissimilar letters are significantly different from each other (p<0.05).



Fig. 4 Initial ion release from the virgin composites.(A) Ca ion release, and (B) P ion release (mean±SD; n=8).



Fig. 5 Ca and P ion recharge and re-release from the rechargeable NACP composites.
(A) Ca ion release, and (B) P ion release (mean±SD; *n*=4). Each recharge cycle was followed by 14 days of continuous release without any recharge during those 14 days. Twelve such cycles of recharge and re-release were tested for each composite, taking 6 months to complete.

initial ion release among the two groups (p>0.1) as both showed increasing ion concentrations with time. At day 70, the rechargeable NACP-DMAHDM composite had Ca ion release of 7.78±0.16 mmol/L and the P ion release was 6.11±0.12 mmol/L.

The results of the twelve cycles of ion recharge and re-release are shown in Fig. 5 (mean \pm SD; n=4): (A) Ca

ion re-release, and (B) P ion re-release. Both composites had continuous ion release after each recharge. For the Ca ion release, the rechargeable NACP-DMAHDM composite had ion release of 0.40 ± 0.07 mmol/L by the end of cycle six, and 0.36 ± 0.02 mmol/L by the end of cycle 12 (p>0.1).

For the P ion release, the rechargeable NACP-



Fig. 6 Ca and P ion re-release concentrations at the last day (day 14) for each cycle of recharge and rerelease.

(A) Ca ion concentration, and (B) P ion release (mean \pm SD; n=4).



Fig. 7 Representative live/dead staining images of 2-day biofilms on composite bars after the 12 cycles of recharge and re-release taking about 6 months to complete.

(A) NACP composite without DMAHDM having live biofilm, (B) NACP-DMAHDM composite with biofilms containing compromised bacteria. Therefore, the 12 cycles of recharge and re-release did not diminish the antibiofilm potency of the NACP-DMAHDM composite.

DMAHDM composite had ion release of 0.32 ± 0.01 mmol/L by the end of cycle six, and 0.30 ± 0.02 mmol/L by the end of cycle 12 (p>0.1). There was a steady-state of ion re-release from cycles 5 to 12, without significant decrease with increasing the number of cycles (p>0.1).

Figure 6 plots the ion re-release at day 14 of each cycle: (A) Ca ion re-release, and (B) P ion re-release (mean \pm SD; n=4). It clearly shows the trend of a moderate decrease in ion release from cycles 1 to 5, and reaching a steady-state of ion re-release from cycles 6 to 12. These results demonstrated that (1) the addition of DMAHDM to the rechargeable NACP composite did not adversely affect the ion recharge and re-release with increasing the number of cycles; (2) The steady-state of ion re-release

indicates continuous ion recharge and re-release to provide long-term ion availability for remineralization.

After 12 cycles of recharge and re-release taking about 6 months in solution to complete, the composite specimens were still able to kill the bacteria, as shown in Fig. 7. The rechargeable-NACP composite with 0% DMAHDM was predominantly covered with green staining with 2-day biofilms. In contrast, the rechargeable NACP-DMAHDM composite was predominantly covered with compromised bacteria with red staining. Comparing the live/dead staining images before and after the 12 cycles (Fig. 2 before cycling, and Fig. 7 after 12 cycles) showed no noticeable differences. This indicates that the DMAHDM composite maintained the potent antibiofilm properties after the 12 cycles of recharge.

DISCUSSION

The present study investigated an antibacterial and remineralizing nanocomposite through the incorporation of DMAHDM and NACP, and evaluated 12 cycles of ion recharge and re-release for the first time, which took 6 months to test. The nanocomposite demonstrated significant antibacterial effects, high levels of initial Ca and P ion release, and long-term ion recharge and rerelease capabilities. Incorporating DMAHDM and NACP into the nanocomposite did not adversely influence the mechanical properties, which were similar to that of a commercial composite. The incorporation of DMAHDM did not compromise the Ca and P ion release and longterm rechargeability of the composite in the 12 cycles, while reducing biofilm quantity, lactic acid production, and CFU counts by 3 to 4 logs.

Resin composites have become increasingly popular due to their minimally invasive approach²⁾, excellent esthetics, and bonding to tooth structure¹⁾. However, the durability of composites can be compromised by their high tendency to accumulate biofilm/plague⁵, resulting in secondary caries and restoration failure^{12,49-51}. Previous studies have shown greater quantities of the cariogenic mutans streptococci adhering to resin composite restorations when compared to amalgam and glass-ionomer⁵¹). Replacement of failed restorations usually occurs due to two main reasons: (1) secondary caries, or (2) restoration fracture. Secondary caries accounts for a greater percentage (87.6%) of all replaced restorations^{3,7,8,10,52)}. Resin composites are more susceptible to developing secondary caries due to the lack of any antibacterial components in their composition, unlike amalgam and glass-ionomer which contain antibacterial metal and fluoride ions, respectively^{4,12,50}. Therefore, it is highly desirable to develop an antibacterial resin composite through the addition of antibacterial components to kill cariogenic bacteria and prevent the development of secondary caries^{10,12)}.

Previous studies have incorporated antibacterial agents, such as fluoride⁵³⁾ and zinc oxide nanoparticles into various dental materials, which provided strong antibacterial effects. However, due to their bacterial selectivity and short-term effects, the search for other antibacterial agents is still a work in progress⁵⁴⁾. Soluble disinfectant such as chlorhexidine⁵⁵⁾, triclosan⁵⁶⁾ and antibiotics, such as vancomycin and metronidazole⁵⁷⁾ were also added to resinous materials to provide antimicrobial properties. However, these materials tend to leach out to the surrounding environment and thus become depleted over time, not only diminishing their antibacterial properties but also resulting in a porous filling material with reduced mechanical properties¹²⁾.

QAMs were developed and copolymerized within the resin to provide long-term antibacterial effects through contact inhibition mechanisms^{12,14,58-61)}. The antibacterial mechanism of action of QAMs is believed

to be through contact-killing. When the cationic agents come in contact with the negatively-charged bacterial cell membrane, they result in an electric imbalance and major changes to the membrane permeability, leading to lysis and subsequent bacterial cell death⁶¹⁾. In addition, QAMs with long alkyl chains can physically pierce the bacterial cell wall, compromising its physical integrity and releasing its cellular contents. The effect of the QAM alkyl chain length (CL) has been evaluated in bonding agents²⁰⁾. Their results showed that DMAHDM with CL of 16 had the strongest antibacterial effect. This was likely due to the dual killing effects on the bacterial cells produced by the electric charge imbalance, as well as the long alkyl chain penetrating the cell membranes²⁰. The composite in our present study used DMAHDM, which provided the aforementioned dual antibacterial mechanisms. In addition, another study demonstrated the antibacterial effect of various DMAHDM concentrations on three-dimensional (3D) biofilm using confocal laser scanning microscopy⁶²⁾. They showed that increasing the concentration of DMAHDM reduced the live biofilm volume and the percentage of live bacteria throughout the biofilm thickness, which was attributed to a triggered programmed cell death (PCD) in the nearby bacteria in the 3D biofilm⁶²⁾. Various chemical compositions of QAMs have been previously developed and incorporated into dental resins including, quaternary ammonium polyethyleneimine (QPEI) nanoparticles 14 and 12-methacryloyloxydodecylpyridinium bromide (MDPB) that showed sustained and long-lasting antibacterial effects that do not diminish over time, without compromising other needed mechanical properties of the material^{15,16,39,58,59)}. A previous study investigated the effect of QAM alkyl chain length of a series of antibacterial monomers on the cytotoxicity of human gingival fibroblasts and odontoblast-like cells in vitro²⁰⁾. The results showed that DMAHDM had a minimal cytotoxicity that matched HEMA and TEGDMA, with significantly less cytotoxicity than Bis-GMA. In addition, they found that when using the cured resin eluents on odontoblast-like cells, the cell viability for the DMAHDM group was similar to the control group using culture medium without any resin eluents²⁰.

In the present study, incorporating 3% DMAHDM into the nanocomposite reduced lactic acid production by S. mutans to one-tenth that of the commercial control composite and reduced CFU counts by 3-4 logs, when compared to other control groups. Lactic acid is the primary acid that accounts for 70% of all bacteriaproduced acids responsible for tooth demineralization⁶³⁾. This renders the new rechargeable NACP-DMAHDM composite to be a powerful tool to combat dental caries. In a previous study, the antibacterial activity of a rechargeable NACP-DMAHDM composite was investigated via a dental plaque microcosm biofilm model and showed a CFU reduction of 3 to 4 logs in S. mutans biofilm alone, as well as in a total microorganism biofilm²⁹⁾. These results were similar to the 3 log CFU reduction achieved in the current study. Another study investigated the antibacterial effects of different DMAHDM concentrations using a multispecies biofilm model consisting of S. mutans, Streptococcus sanguinis (S. sanguinis) and Streptococcus gordonii (S. gordonii), and showed 2-3 logs in biofilm CFU reduction²³⁾. Lin et al. investigated the antibacterial effects of DMAHDMcontaining composite against biofilms with 1, 3, 6 and 9 species of periodontal pathogens. Although their results indicated a slightly decreasing killing-efficacy of DMAHDM with increasing the number of bacterial species, the composite was still able to reduce the ninespecies biofilm CFU by nearly 44 folds⁶⁴⁾. These results indicate that the DMAHDM-containing resin is effective in inhibiting both single species and multispecies biofilms, which warrant further study. In a previous study, the effects of salivary pellicles on bonding agents containing dimethylaminododecyl methacrylate (DMADDM) on biofilm inhibition were investigated. Indeed, pre-coating of salivary pellicles on the resins moderately reduced the antibacterial effects of DMADDM. However, the DMADDM-containing bonding agent resin still reduced the biofilm CFU by nearly two orders of magnitude even with the presence of salivary pellicle coating on the resin surface⁶⁵⁾. In our current study, DMAHDM with a longer alkyl chain than DMADDM was used, hence DMAHDM had a stronger antibacterial activity than DMADDM. Therefore, it is expected that the DMAHDM resin would have a strong antibacterial function even with salivary pellicle coating on the resin surface. Further studies are needed to investigate a more complex multispecies biofilm model with salivary protein pellicle coating to better assess the antibacterial activity of the novel ionrechargeable NACP-DMAHDM composite.

QAMs have antibacterial effects against a wide spectrum of microbes, including bacteria and fungi¹⁶⁾. A limited number of studies investigated the possible development of antibacterial drug-resistance against QAMs. A recent study addressed the concerns of persisters in S. mutans biofilm when using DMAHDM⁶⁶. The tolerance of surviving S. mutans persisters in the initial population to DMAHDM was not transferred to the subsequent generations⁶⁶⁾. DMAHDM-induced S. *mutans* persister biofilms could be totally eradicated by using higher concentrations of DMAHDM⁶⁶⁾. In addition, no increase in minimum inhibitory concentration (MIC) values was detected between S. mutans parental strain and S. mutans persisters induced by DMAHDM, indicating that the surviving bacterial strain was not a resistant strain⁶⁶⁾. In another study by Kitagawa et al., the development of bacterial resistance using S. mutans and Enterococcus faecalis (E. faecalis) after repeated exposures to cationic antimicrobial agents was assessed. They showed that after 10 passages of exposure to cationic biocides, S. mutans and E. faecalis did not exhibit any resistance to MDPB, a QAM antibacterial agent⁶⁷⁾. Therefore, while these studies indicated no oral bacterial resistance to QAMs, further studies are needed on the possible development of antibacterial drugresistance against QAMs after longer term exposures.

NACP has shown its smart ability to release high levels of Ca and P ions especially at acidic pH environments where demineralization of tooth structure occurs, without negatively affecting the mechanical properties^{28,45)} of the material, and without the presence of apatite seed crystallites to initiate the remineralization process⁶⁸⁾. Previous studies reported the ability of nanocomposite containing NACP and a poly (amido-amine) (PAMAM) dendrimer as nucleation template to remineralize the pre-demineralized human dentin in a cyclic artificial saliva/lactic acid challenge environment and increase dentin hardness to match the hardness of pre-demineralized dentin^{69,70}. Utilizing a human-in situ model, a NACP containing composite was also able to inhibit caries formation through the release of high levels of Ca and P ions that increased at lower cariogenic pH levels⁴⁵⁾. When microradiographs were taken, less carious lesions were observed in enamel around the NACP containing composite, when compared to commercial control⁴⁵⁾. Recently, a rechargeable NACPcomposite was developed and demonstrated Ca and P ion recharge and re-release capabilities^{29,30}. However, the rechargeability of the NACP-composite was tested over a short duration. The present study developed an antibacterial and rechargeable NACP-composite and investigated its long-term ability to recharge and rerelease Ca and P ions.

The results of the current study showed that incorporating 20% NACP into the composite released high levels of Ca and P ions and was successfully recharged with Ca and P ions over 12 cycles of ion recharge and re-release, allowing the composite to serve as a Ca and P ions reservoir capable of providing longterm remineralization. Previous studies on resin-based Ca-P cements released Ca and P ions at levels of 0.3 and 0.05 mmol/L, respectively, which were shown to be sufficient to remineralize tooth lesions⁴⁶⁾. The present study incorporated NACP with a much smaller particle size of 116 nm and a higher surface area of $17.76 \text{ m}^2/\text{g}$ when compared to $0.5 \text{ m}^2/\text{g}$ of traditional CaP particles. The NACP-composite in the present study released 0.36 and 0.3 mmol/L of Ca and P ions, respectively, after 12 cycles of recharge and re-release. These findings indicate that the levels of Ca and P ion releases exceeded the needed levels for remineralization as shown by previous studies⁴⁶⁾. Therefore, the present rechargeable composite possessed a long-term ion-release and remineralization capability.

The EBPM resin used in the present study is responsible for the rechargeability of the composite, due to the presence of the PMGDM monomer. PMGDM contains active carboxylate groups that can chelate with Ca or P ions in recharge solutions at pH 7. During an acidic attack, the bond between the PMGDM and Ca ions can break, releasing these ions into the surrounding surfaces. After the initial Ca and P ion release, the sites previously occupied by the Ca and P ions become vacant to accept incoming ions from the recharge solution. This Ca and P chelation ability allows for ion release and reuptake by the EBPM resin³⁰. The recharge solution used in the present study simulates a potential mouthwash containing Ca and P ions that can be used by the patient for 3 min/day for one day, which can provide two weeks of continuous ion release. This could be especially beneficial for high caries risk patients.

The incorporation of NACP and DMAHDM into the composite produced antibacterial and remineralization capabilities without compromising the flexural strength and modulus of elasticity of the material. It is important for composite to be strong and capable of withstanding high chewing forces, especially when used in Class I and Class II restorations. The uncompromised flexural strength of the NACP-DMAHDM composite can be attributed to the addition of the reinforcing glass particles. Surface roughness is another important property for resin-composites, as rough restoration surfaces retain more plaque, cause superficial staining, and gingival inflammation; and thus affect the whole clinical performance of the dental restoration^{71,72}. Incorporating DMAHDM and NACP into the composite showed surface roughness values similar to that of commercial control.

The rechargeable NACP-DMAHDM composite retained its antibacterial activity after 12 cycles of recharge, maintaining the bacterial killing ability. This is attributed to the immobilization of DMAHDM within the resin, thus providing long-term antibacterial effects. Our findings are consistent with findings of previous studies where the antibacterial effects of DMAHDM were maintained after one year of water aging⁷³⁾. DMADDM, a QAM with a shorter alkyl chain showed a similar trend in maintaining its antibacterial properties after 6 months of water-aging⁷⁴). Hence, this bioactive and therapeutic composite can shift the clinical practice into a more conservative and minimally invasive practice by killing remaining bacteria and remineralizing demineralized tooth lesions due its antibacterial properties, remineralization capability, and long-term recharge and re-release effect. Further studies are needed to investigate caries-inhibition using an in-situ model simulating clinical applications to evaluate the efficacy of the rechargeable NACP-DMAHDM composite.

CONCLUSION

This study investigated a novel DMAHDM-NACP composite with strong antibacterial and ion recharge capabilities, demonstrating long-term Ca and P ion release and remineralization potential with high levels of Ca and P ion release even after 6 months of recharge and re-release. DMAHDM reduced S. mutans biofilm CFU by nearly 4 logs, without adversely affecting the composite mechanical properties and ion release and rechargeability. Additionally, this rechargeable composite provided substantial recharge and sustained release of Ca and P ions for potential long-term remineralization and caries-reduction effects. Both the rechargeable NACP composite and the rechargeable NACP-DMAHDM composite presented similar levels of Ca and P ion release, recharge and re-release, that reached a steady-state as the number of recharge

and re-release cycles increased. Furthermore, the rechargeable NACP-DMAHDM composite was able to maintain its potent antibacterial effect even after 6 months of recharge and re-release. Therefore, the long-term rechargeable NACP-DMAHDM composite with much less biofilm growth and lactic acid production is promising to inhibit caries, prevent demineralization, and enhance remineralization of tooth structures.

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